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Hemolymph Juvenile Hormone Binding Protein of Fifth Instar Larvae of *Bombyx mori* L.: Identification and Purification

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Juvenile hormone binding protein was identified in the hemolymph of fifth instar larvae and purified using column chromatography. Hemolymph was mixed with [3H] JH-III and electrophoresed on 6% NON-SDS gel, indicating that radioactivity peak appears at Rf value of 0.55. Gel filtration showed two radioactivity peaks equivalent to bound and free [3H]JH-III, respectively. JHBP was purified from hemolymph through gel filtration (Sephadex G-100), anion exchange chromatography (DEAE Sepharose CL-6B), chromatofocusing chromatography (PBE 94) and preparative electrophoresis.

KEY WORDS: Juvenile Hormone Binding Protein, Protein Purification, *Bombyx mori*

Juvenile hormone (JH) which is synthesized by fat body and released into hemolymph and acts at various target cells was known to play very important role in molting, metamorphosis, diapause, and reproduction along with ecdysone and also its amount was to be controlled by various proteins in hemolymph (de Kort and Granger, 1981). That is, it appears throughout whole developmental stages and general esterase degrades free JH only while JH specific esterase which is synthesized in large amounts in late last instar stage and in some specific stage destroys both bound JH (combined with JHBP) and free JH. These two kinds of esterases lower the concentration of JH in hemolymph whereas JHBP prevents JH from being degraded by general esterase through specific binding with JH and also transports JH to various target cells (Sanburg *et al.*, 1975; Rudnicka *et al.*, 1979). Also, real concentration of JH becomes very low in hemolymph because normal physiological concentration of JH is low and also JH attached nonspecifically to hydrophobic surface such as cell membrane and muscle. However, this problem is solved by combining with JHBP (Goodman and Chang, 1985).

JHBP in hemolymph was first reported by Whitmore and Gilbert (1972) and then has been intensively studied in various insect species. In general, JHBP can be divided into two kinds depending upon the affinity to JH (Goodman and Chang, 1985). Low affinity and high molecular weight JHBP was found in Lepidoptera with high affinity and low molecular weight JHBP but its function is still uncertain. High affinity JHBP can be again subdivided into two kinds according to molecular weight. Low molecular weight (25-35 kD) JHBP was found in lepidopteran larvae including *manduca sexta* (Kramer *et al.*, 1976a; Lenz *et al.*, 1986; Prestwich *et al.*, 1987), *Galleria mellonella* (Rudnicka *et al.*, 1979), *Diatraea grandiosella* (Turunen and Chippendale,

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1979, 1981; Dillwith *et al.*, 1985; Lenz *et al.*, 1986), *Diatraea crambidoides, Diatraea saccharalis, Ostrinia nubilalis, Homoeosoma electellum* (Lenz *et al.*, 1986) and *Hyphantria cunea* (Lee and Kim, 1993) whereas high molecular weight JHBP in non-lepidopteran species including *Leucophaea maderae* (Engelmann *et al.*, 1988; Koeppe *et al.*, 1988), *Diploptera punctata* (King and Tobe, 1988), *Periplaneta americana* (de Kort *et al.*, 1984), *Locusta migratoria* (de Kort *et al.*, 1984; Koopmanschap and de Kort, 1988), *Leptinotarsa decemlineata* (de Kort *et al.*, 1983, 1984; de Kort and Koopmanschap, 1987), *Sarcophaga bullata* (Mellaert *et al.*, 1985) and *Drosophila melanogaster* (Shemshedini and Wilson, 1988). Also, JHBP which acts as JH receptor was found in other tissues except for hemolymph and it was known to have high affinity (Klages *et al.*, 1980; Engelmann, 1981; Mellaert *et al.*, 1985; Wisniewski *et al.*, 1988; Palli *et al.*, 1990; Shemshedini *et al.*, 1990).

Present work is to identify JHBP in larval hemolymph of *Bombyx mori* and also to purify them using gel filtration, anion exchange chromatography, chromatofocusing chromatography and preparative electrophoresis.

Materials and Methods

Chemicals

All reagents used in the present work were of reagent grade and purchased as follows: [10-^{3}H] JH-III (sp. act. 17 Ci/mmol) (New England Nuclear); diethyl p-nitrophenyl phosphate (paraoxon), polyethylene glycol 20,000, Sephadex G-100, sodium dodecyl sulphate, 2-mercaptoethanol, phenylthiourea, acrylamide, bis-acrylamide, glycine (Sigma Chemical Co.); DEAE Sepharose CL-6B, polybuffer exchanger 94, polybuffer 74 (Pharmacia); SDS-PAGE molecular weight standards (BioRad); sodium azide, ethyl alcohol (Merck); Omnifluor (Dupont)

Insects and collection of hemolymph

Bombyx mori (Baekokjam, Jam 123 × Jam 124) were obtained from Sericultural Experiment Station, RDA and reared on artificial diet at 27 ± 1°C and 70 ± 10% R.H. under the photoperiod of 16L:8D. Hemolymph was collected from one or two day old fifth instar larvae by cutting legs and put into cold test tube which contains small amounts of phenylthiourea. The hemolymph was centrifuged at 10,000 g for 10 min and the supernatant was stored at −70°C until used.

Analytical electrophoresis

Hemolymph reacted with 10^{-7} M [^{3}H]JH-III was electrophoresed on 6% gel according to Davis (1964) to confirm the presence of JHBP. After electrophoresis, one gel was stained in 0.25% Coomassie brilliant blue whereas other gel sliced at 3 mm thickness. Each slice was put into 10 ml of scintillation cocktail (toluene 2l/Triton X-100 1l/Omnifluor 12g) and continuously shaked for 20 hrs. The radioactivity of each slice was measured by liquid scintillation counter (Beckman LS 100 C). Samples in each purification step were electrophoresed on 10% SDS PAGE gel as described by Laemmli (1970) to confirm purity of JHBP.

Analytical gel filtration

Hemolymph (1 ml) of early fifth instar larvae was mixed with the equal volume of 10 mM Tris-HCl/50 mM NaCl, pH 7.3 (TN buffer) containing 2×10^{-4} M paraoxon and equilibrated at 4°C for 1 hr and then reacted with 10^{-7} M [^{3}H]JH-III for 30 min. This sample was equilibrated with TN buffer and then eluted from calibrated Sephadex G-100 column (1.9 × 95 cm) at the flow rate of 20 ml/hr with 3 ml per fraction. Protein concentration in each fraction was measured at 280 nm. Also, 0.1 ml per fraction was mixed with 10 ml of scintillation cocktail for the measurement of radioactivity. Standard proteins used for calibration were alcohol dehydrogenase (150, 000), bovine serum albumin (66,000), carbonic anhydrase(29,000) and cytochrome c (12,400) and void volume was measured using Blue dextran 2000.

DCC binding assay

Dextran coated charcoal (DCC) binding assay was carried out as described by Kramer *et al.*

(1976b) with slight modification to confirm JH binding capacity of hemolymph and column fraction. Activated charcoal was washed with 1 N HCl, H_2O and 1% (w/v) $NaHCO_3$ in order and then with H_2O until neutralized. Washed activated charcoal was completely dried and 1 g of dried charcoal and 0.5 g of dextran were added to 100 ml of 10 mM Tris/1.5 mM EDTA/3mM NaN_3 (pH 7.3) and stirred at 4°C for 10 hr. This was washed with above buffer solution five times and diluted two-fold for use. DCC assay was conducted in the following order. Appropriate amount of [^{3}H] JH-III dissolved in ethanol was put in test tube and evaporated with nitrogen gas. One hundred μl of protein solution was added to the hormone and incubated at 4°C for 30 min. One hundred μl of charcoal solution was again added to this mixture with constant stirring and centrifuged at 10,000 g for 1 min. At this time, JH bound to JHBP remains at supernatant whereas free JH was sunk owing to combining with DCC. One hundred μl was taken from supernatant containing radiolabeled hormone and mixed with 10 ml of scintillation cocktail for the measurement of radioactivity.

Preparative gel filtration

As the first step of JHBP purification, gel filtration was performed. Hemolymph (3 ml) of early fifth instar larvae was equilibrated with the equal volume of TN buffer containing 2×10^{-4} M paraoxon and then incubated with 10^{-7} M [^{3}H]JH-III for 30 min. This sample was eluted from column (1.9 × 95 cm) on Sephadex G-100 at the flow rate of 20 ml/hr with 6 ml per fraction. One hundred μl of each fraction was used for the measurement of radioactivity. These processes were repeated five times and fractions with high radioactivity were concentrated and dialyzed against TN buffer for 10 hrs for anion exchange chromatography.

Anion exchange chromatography

Above sample was applied to the column(1.8 × 14 cm) of DEAE Sepharose CL-6B previously equilibrated with TN buffer. Unbound proteins not combined with resin were eluted with 100 ml of TN buffer while proteins bound to resin with TN buffer containing 0.05-0.35 M NaCl gradient (200 ml) at the flow rate of 25 ml/hr with 3 ml per fraction. Fractions with high radioactivity were concentrated and dialyzed against 0.025 M bis-tris buffer (pH 7.3) for chromatofocusing chromatography.

Chromatofocusing chromatography

Column (0.9 × 21 cm) was packed with polybuffer exchanger 94 (PBE 94) at the flow rate of 40 ml/hr and equilibrated with 150 ml of bis-tris buffer (pH 7.3) for chromatofocusing chromatography. Column was washed with 5 ml of 0.025 M polybuffer 74 (pH 5.0) and then sample was eluted with 0.025 M polybuffer 74 at the flow rate of 18 ml/hr to be pH gradient(pH 5-pH 7) with 3 ml per fraction. Fractions with high radioactivity were concentrated and dialyzed against TN buffer for the next purification step.

Preparative electrophoresis

Above sample was electrophoresed on 6% preparative slab gel. After electrophoresis, one gel was sliced for the measurement of radioactivity and the part of other gel equivalent to the segment with high radioactivity was cut and electro-eluted at 200 V for 2 hrs for protein extraction.

Results

Identification of JHBP

Hemolymph of early fifth instar larvae incubated with [^{3}H]JH-III was electrophoresed on 6% gel to confirm the presence of protein specifically bound to JH. After electrophoresis, gel was sliced for the measurement of radioactivity. In hemolymph previously incubated with 10^{-4} M paraoxon (inhibitor of general esterases), one radioactivity peak appeared at Rf value of 0.55 but in hemolymph not treated, one peak appeared near the tracking dye (Fig. 1). Also, hemolymph incubated with 10^{-4} M paraoxon was eluted from Sephadex G-100 column calibrated with standard protein and radioactivity of each fraction was measured. Two peaks were shown but first peak only appeared by DCC assay (Fig. 2). JHBP fraction eluted from Sephadex G-100 column was

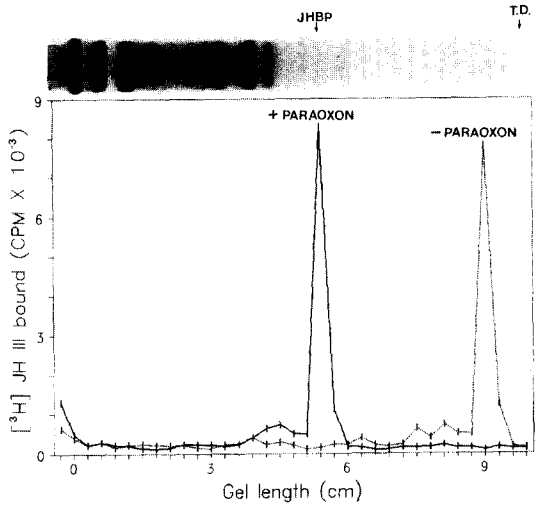

Fig. 1. Electropherogram and distribution of radioactivity in a native gel of hemolymph treated with 10^{-7}M [^{3}H]JH-III in the presence (——) or absence (··· ···) of 10^{-4}M paraoxon.

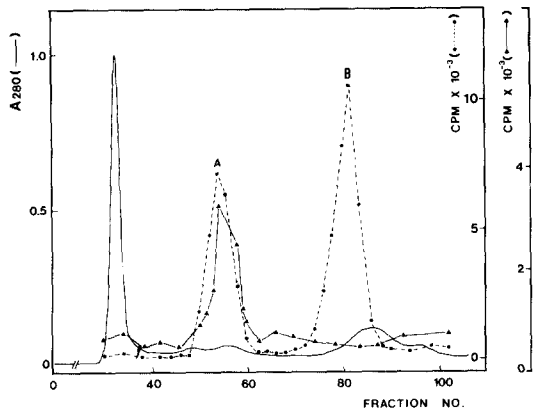

Fig. 2. Column chromatographic separation of hemolymph on a Sephadex G-100 column (1.9 × 95 cm). Hemolymph treated with 10^{-4}M paraoxon was diluted with 10mM Tris/50 mM NaCl (pH 7.3, TN buffer) by two-fold and then incubated with 10^{-7}M [^{3}H] JH-III. JH binding was measured directly by counting of 100 μl aliquots of each fraction (● ····· ●) or by DCC assay (▲——▲). A; bound peak B; unbound peak.

electrophoresed and hemolymph incubated with 10^{-4} M paraoxon and [^{3}H]JH-III was also electrophoresed and radioactivity of both gels each was measured. Two gels showed one radioactivity peak at the same location (Fig. 3).

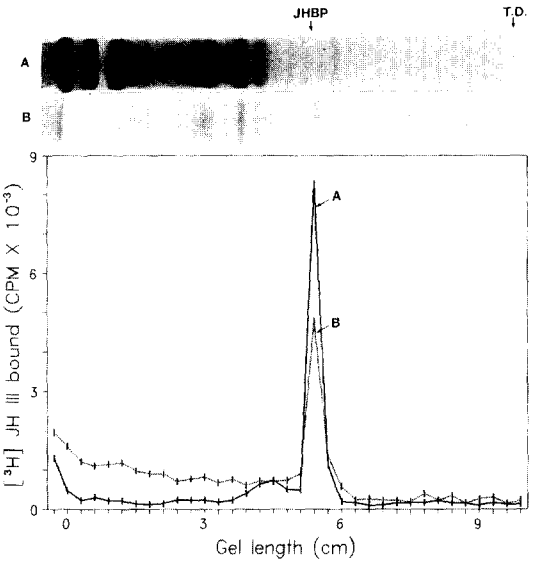

Fig. 3. Electropherogram and distribution of radioactivity in a native gel of hemolymph (A) and radioactive fractions from gel filtration (B) treated with 10^{-7} M [^{3}H]JH-III in the presence of 10^{-4}M paraoxon.

Purification of JHBP

JHBP was purified from hemolymph of early (1-2 day old) fifth instar larvae when 30K protein was barely synthesized. As the first purification step, gel filtration using Sephadex G-100 was carried out. Hemolymph (3 ml) treated with 10^{-4} M paraoxon for 1 hr was incubated with [^{3}H]JH-III and then diluted with the same volume of TN buffer (10% glycerol, v/v). Storage protein occupying most of hemolymph protein in fifth instar larvae was separated as the first protein peak and JHBP was eluted with 30K protein (component 1) from 330 ml of elution volume (Fig. 4). Fractions containing JHBP were applied to anion exchange chromatography to remove 30K protein. As shown in Fig. 5, 30 K protein was eluted as unbound peak (A) whereas JHBP separated at NaCl concentration of 0.18 M. To determine the location of JHBP on SDS PAGE gel, radioactive fractions obtained through ion exchange chromatography were electrophoresed, indicating that JHBP band appeared near molecular weight of 32kD (Fig. 6). To purify JHBP further, this sample was subjected to

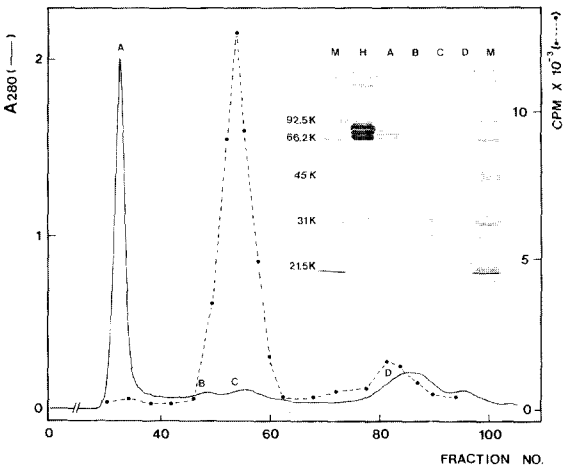

Fig. 4. Gel filtration of hemolymph incubated with 10^{-7} M [^{3}H]JH-III in the presence of 10^{-4} M paraoxon on a column of Sephadex G-100 (1.9 × 95 cm). JH binding was measured directly by counting of 100 μl aliquots of each fraction.

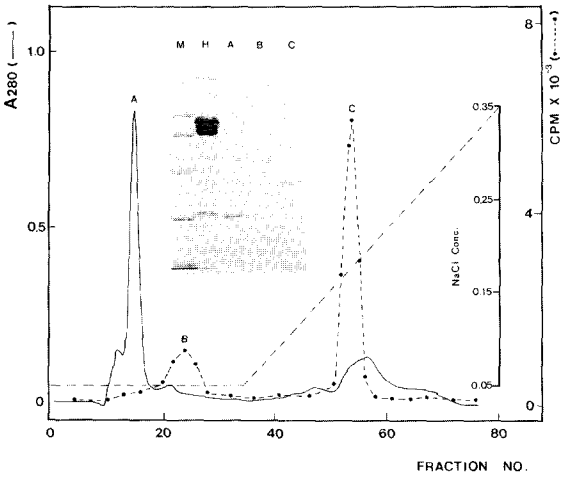

Fig. 5. Ion exchange chromatography of radioactive fractions (56-60) from gel filtration on a column of DEAE Sepharose CL-6B equilibrated with TN buffer. Fractions containing JHBP were concentrated, dialyzed and applied to the column. Unbound proteins were eluted with TN buffer and bound proteins were eluted with a gradient of 0.05 M to 0.35 M NaCl in the same buffer.

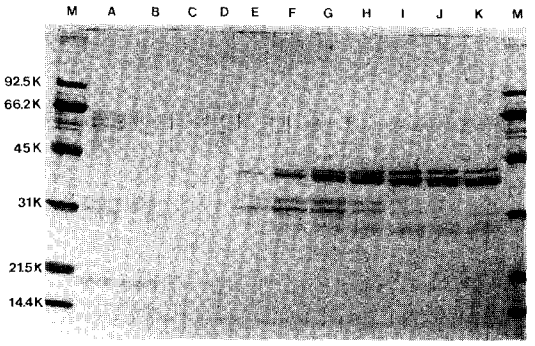

Fig. 6. SDS-PAGE of fractions (48-58) from ion exchange chromatography. M; standard molecular weight marker, A-K; fractions 48-58, respectively.

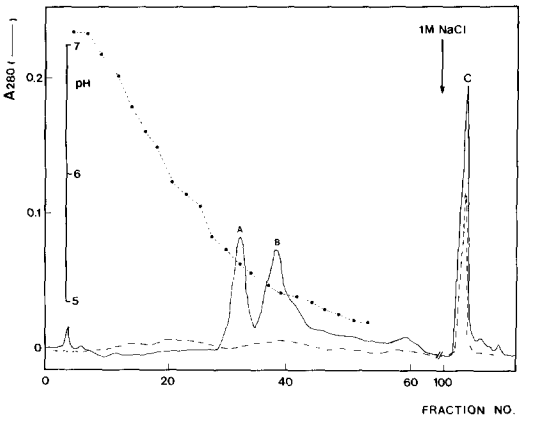

Fig. 7. Chromatofocusing chromatography of radioactive fractions (52-55) from ion exchange chromatography on a column of polybuffer exchanger 94 (PBE 94) equilibrated with 0.025 M bis-tris buffer (pH 7.3). Fractions containing JHBP were concentrated, dialyzed against the equilibration buffer, incubated with 10^{-7} M [^{3}H]JH-III and applied to the column. The column was eluted with 0.025M polybuffer 74 and JH binding was measured directly by counting of 100 μl aliquots of each fraction (- - -).

chromatofocusing chromatography. Two protein peaks appeared in the pH range of 5-7 and one peak with high radioactivity in 1 M NaCl fraction (Fig. 7). However, since this radioactive fraction showed 3-4 different bands on electrophoretic gel, preparative electrophoresis was performed on 6% NON-SDS PAGE gel to remove contaminants. After electrophoresis, gel was sliced and

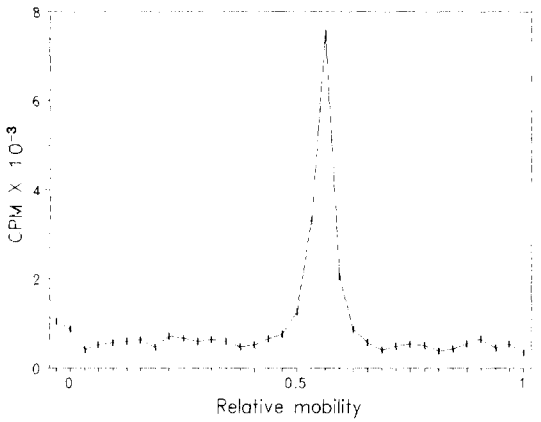

Fig. 8. Preparative electrophoresis of radioactive fractions from chromatofocusing chromatography. Fractions containing JHBP were concentrated, dialyzed against Tris-HCl buffer (pH 6.8) and electrophoresed in a 6% native polyacrylamide gel. After electrophoresis, part of the gel was sliced and the radioactivity in each segment was measured.

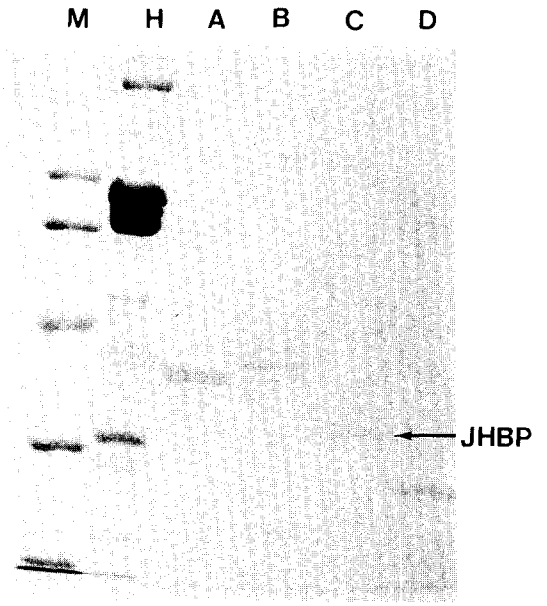

Fig. 9. SDS-PAGE of purified JHBP. Radioactive fractions from chromatofocusing chromatography were concentrated, dialyzed against 20 mM Tris-HCl buffer (pH 6.8), incubated with 10^{-7} M [^{3}H]JH-III and electrophoresed natively. After electrophoresis, part of the gel was sliced and its radioactivity was measured. The other gels which have Rf value of 0.45 to 0.6 were subjected to electro-elution separately. M; standard molecular weight marker, A-D; eluted proteins which have Rf value of 0.45, 0.5, 0.55 and 0.6, respectively.

radioactivity of each slice measured. Single peak appeared at Rf value of 0.55 (Fig. 8). This protein was electro-eluted and again electrophoresed, confirming single band of JHBP (Fig. 9).

Discussion

JH homologues such as JH II (Meyer *et al.*, 1968) and JH III (Judy *et al.*, 1973) were discovered in order since chemical structure of JH I (Roller *et al.*, 1967) has been determined in late 1960s. Along with these facts, JHBP specifically bound to these hormones has been actively studied in many insect species. The function of JHBP was summarized as follows: (1) JHBP prevents JH from being degraded by general esterases. (2) it transports JH to various target tissues. (3) it acts as reservoir available immediately when needed at target cells (Goodman and Chang, 1985; Goodman, 1990).

As the first step of JHBP study, NON-SDS PAGE or gel filtration was carried out to determine the presence of JHBP in hemolymph (Klages and Emmerich, 1979; Turunen and Chippendale, 1981; de Kort *et al.*, 1983; Dillwith *et al.*, 1985; Lenz *et al.*, 1986). In the present work with *Bombyx mori*, hemolymph incubated with [^{3}H]JH-III was electrophoresed on 6% NON-SDS PAGE gel to determine the presence of JHBP. As shown in Fig. 1, radioactivity peak appeared at Rf value of 0.55 but corresponding band could not be confirmed on polyacrylamide gel. This fact indicates that JHBP specifically bound to JH is present in larval hemolymph but in very small amount so that it can not be detected with Coomassie brilliant blue staining. Above sample eluted from Sephadex G-100 showed two radioactive peaks but first peak only appered by DCC assay (Fig. 2). Also, hemolymph incubated with [^{3}H]JH-III was treated with DCC before gel filtration to remove free JH. At this time, first peak remained but second peak disappeared. This result indicated that first peak was due to JHBP but second peak was owing to free JH. According to update data, two kinds of JHBP, that is, low

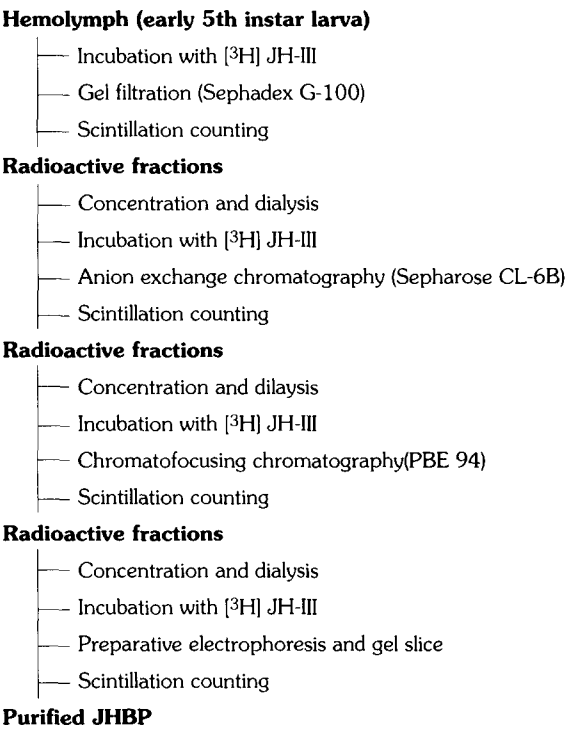

Fig. 10. Flow diagram of the purification of JHBP from *Bombyx mori* hemolymph

affinity and high molecular weight JHBP and high affinity and small molecular weight JHBP were generally found in lepidopteran larvae (Klages and Emmerich, 1979; Rudnicka *et al.*, 1979; Turunen and Chippendale, 1981; Lee and Kim, 1993). As shown in Fig. 2, low affinity and high molecular weight JHBP could not be confirmed in *Bombyx mori*.

High affinity and low molecular weight JHBP has been extensively studied in *Manduca sexta*. Also, purification of JHBP was first performed from larval hemolymph of the insect. JHBP was purified using gel filtration, ion exchange chromatography, and preparative isoelectric focusing (Kramer *et al.*, 1976b) or through affinity chromatography using JH II as immobilized ligand (Goodman and Goodman, 1981). After that, JHBP has been also purified in the other insect species (Dillwith *et al.*, 1985; Wang *et al.*, 1989; Prestwich and Atkinson, 1990).

In *Bombyx mori*, 30 K proteins composed of four components (1,2,3 and 4) are intensively synthesized and released into hemolymph after middle fifth instar larval stage, occupying most of hemolymph proteins with storage proteins (Izumi *et al.*, 1981). Since components 3 and 4 could not be separated from JHBP through gel filtration and anion exchange chromatography, these components cause main problem in purifying JHBP of *B. mori*. In the present experiment with *B. mori*, JHBP was purified from hemolymph of early (1-2 day old) fifth instar larvae when 30 K protein was not intensively synthesized. As the first purification step, gel filtration using Sephadex G-100 was carried out to remove storage protein occupying most of larval hemolymph proteins. As shown in Fig. 4, most of storage proteins were eluted through first protein peak but radioactive fractions containing JHBP were mostly occupied by component 1 which was little by little synthesized in early fifth instar larval stages. JHBP could not be recognized by Coomassie brilliant blue staining. Therefore, anion exchange chromatography was carried out to remove

component 1. Component 1 was eluted at unbound fraction whereas JHBP at NaCl concentration of 0.18 M. At this time, another radioactive peak appeared around fraction No 25. However, this peak disappeared if hemolymph incubated with [^{3}H]JH-III is treated with DCC for 2 min before application to column or undergoes DCC assay during fraction analysis. These facts indicated that this radioactive peak was due to free [3H]JH-III unbound to JHBP. Also, radioactive fractions were electrophoresed at the same time to confirm JHBP band on SDS PAGE gel and JHBP-like band could be confirmed around 32 kD (Fig. 6). In chromatofocusing chromatography, however, two protein peaks appeared in the pH gradient of 7-5. At this time, unbound protein was eluted with NaCl as a third peak and this peak showed high radioactivity (Fig. 7). However, since this peak also showed 3-4 bands on SDS PAGE gel, preparative electrophoresis was carried out. Four different proteins appeared in the Rf range of 0.45-0.60 and JHBP is purified as a single band at Rf value of 0.55.

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누에나방(***Bombyx mori*** L.)의 5령유충 혈림프의 유약호르몬 결합단백질(Juvenile hormone binding protein): 확인 및 정제

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누에나방(*Bombyx mori* L.)의 5령유충 혈림프에 유약호르몬 결합단백질(juvenile hormone binding protein, JHBP)이 존재함을 확인한 후 이를 column chromatography를 사용하여 정제하였다. [^{3}H]JH-Ⅲ와 반응시킨 혈림프를 6% gel에서 NON-SDS PAGE를 실시한 결과 Rf 0.55 위치에서 radioactive peak가 확인되었으며 gel filtration을 실시한 결과 bound 및 free [^{3}H]JH-Ⅲ에 해당하는 2개의 radioactivity peak가 확인되었다. Gel filtration(Sephadex G-100), anion exchange chromatography(DEAE Sepharose CL-6B), chromatofocusing chromatography (PBE 94) 및 preparative electrophoresis를 통하여 혈림프 JHBP를 순수정제하였다.